FORM PTO-1390 (REV. 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: EX96002-US

U.S. APPLICATION NO. (If known See 37 C F R 1 5): 09/125887

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR97/00435	12 March 1997	14 March 1996

TITLE OF INVENTION

RECOMBINANT ADENOVIRAL VECTORS FOR HUMAN TUMOUR GENE THERAPY

APPLICANT(S) FOR DO/EO/US

Thierry BOON-FALLEUR, Marie-Thérèse DUFFOUR, Heidi HADDADA, Christophe LURQUIN, Michel PERRICAUDET, Catherine UYTTEN-HOVEGHESQUIERE and Guy WARNIER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney, unsigned.
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Submission of Computer Readable Sequence Listing; Paper Copy Sequence Listing; and CRF (disk) copy Sequence Listing.

CERTIFICATION UNDER 37 CFR 1.10

"Express Mail" Mailing Number	Date of Deposit
GB840710365US	August 27, 1998

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231, Attn. EO/US

(Type or print name of person mailing paper)	(Signature of person mailing paper)
Paula L Dickey	Paula L. Dickey

U.S. APPLICATION NO. (If Known, see C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	PCT/FR97/00435	EX96002-US

17. [X] The following fees are submitted:

	CALCULATIONS	PTO use only
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....$ 930.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) .....$ 720.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..$ 790.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid USPTO.....$1070.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 98.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 930.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	21 - 20 =	1	X $ 22.00	$ 22.00	
Independent Claims	2 - 3 =	0	X $ 82.00	$	
Multiple dependent claim(s) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 952.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 952.00	
Processing fee of $130.00 for furnishing the English translation later the [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 952.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 952.00	
				Amount to be refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fee is enclosed.

b. [X] Please charge my Deposit Account No. 18-1982 in the amount of $952.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-1982. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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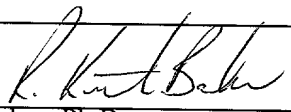
Signature 

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Name

38,799
Registration Number

Date 8/27/98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Boon-Falleur, et al.	Group Art Unit:
Serial No.:	To Be Assigned	Examiner:
	U.S. National Stage of PCT/FR97/00435	
Filed:	Concurrently Herewith	

For: Recombinant Adenoviral Vectors For Human Tumour Gene Therapy

To: Assistant Commissioner For Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING (37 CFR § 1.10)

GB840710365US
"Express Mail" Mailing Number

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Paula L. Dickey
(Type or print name of person mailing paper)

Paula L. Dickey
(Signature of person mailing paper)

PRELIMINARY AMENDMENT

Please enter the following amendment to the English Translation of the International Application before examining this application.

In the Specification

Please delete the Sequence Listing on pages 44-46, and insert the attached SUBSTITUTE SEQUENCE LISTING (page 1) after the Abstract.

In the Claims

Please cancel claims 1-19 without prejudice.

Please add the following new claims 20-40:

20. A replication defective recombinant adenovirus comprising a nucleic acid encoding a tumour-specific antigen.

21. The adenovirus according to claim 20, wherein the tumour is a human tumour.

22. The adenovirus according to claim 20, wherein the tumour is a melanoma.

23. The adenovirus according to claim 20, wherein the nucleic acid encodes a fragment of an antigen specific to a human melanoma, said fragment comprising the portion of the antigen presented to cytotoxic T lymphocytes in combination with MHC-I molecules.

24. The adenovirus according to claim 20, wherein the nucleic acid encodes a protein selected from the group consisting of Mage-1, Mage-3, Bage, Rage and Gage, or an antigenic peptide therefrom.

25. A replication defective recombinant adenovirus comprising a nucleic acid encoding a peptide fragment of Mage-1 or Mage-3, wherein the fragment comprises the portion of Mage-1 or Mage-3 presented to cytotoxic T lymphocytes.

26. The adenovirus according to claim 25, comprising the sequence SEQ ID No. 1.

27. The adenovirus according to claim 26, comprising the sequence lying between residues 55 and 82 of SEQ ID No. 1.

28. The adenovirus according to claim 25, comprising the sequence SEQ ID No. 2.

29. The adenovirus according to claim 20, wherein said adenovirus is selected from the group consisting of human serotype Ad2 and Ad5.

30. The adenovirus according to claim 20, wherein the adenovirus is a canine adenovirus.

31. The adenovirus according to claim 20, further comprising a deletion in the E1 region.

32. The adenovirus according to claim 31, further comprising a deletion in the E4 region.

33. The adenovirus according to claim 20, wherein the nucleic acid is inserted into the E1, E3 or E4 region of the adenovirus genome.

34. A pharmaceutical composition comprising an adenovirus according to claim 20.

35. A composition comprising cells infected with an adenovirus according to claim 20.

36. The composition according to claim 35, wherein said cells are antigen presenting cells.

37. A method of preparing cytotoxic T cells specific for a tumour antigen, the method comprising contacting a cytotoxic T cell precursor with a population of cells infected with an adenovirus according to claim 20.

38. A method of preparing cytotoxic T cells specific for a tumour antigen, the method comprising administering to a patient an adenovirus according to claim 20.

39. The method according to claim 37, wherein the tumour is a melanoma.

40. The method according to claim 38, wherein the tumour is a melanoma.

REMARKS

Applicants submit a SEQUENCE LISTING in compliance with the requirements of 37 CFR §§ 1.821-1.825. No new matter has been added.

Claims 1-19 have been cancelled and rewritten as new claims 20-40, in order to conform with US patent practice. The claims are fully supported by the claims as filed and by the specification. No new matter has been added. Early and favorable examination of this application is earnestly solicited.

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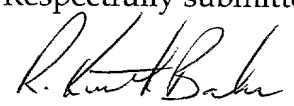
Dated: August 27, 1998

Respectfully submitted,



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<120> RECOMBINANT ADENOVIRAL VECTORS FOR HUMAN TUMOUR GENE THERAPY

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RECOMBINANT ADENOVIRAL VECTORS FOR HUMAN TUMOUR GENE THERAPY

The present invention relates to a method for the treatment of human tumours by gene therapy. It relates especially to defective recombinant viruses carrying a sequence coding for an antigen specific to human tumours, and to their use for the preventive or curative treatment of human tumours and also for generating specific CTL in vitro or ex vivo. It also relates to pharmaceutical compositions containing these viruses, in particular in injectable form.

Gene therapy consists in correcting a deficiency or an abnormality by introducing genetic information into the affected cell or organ. This information may be introduced either in vitro into a cell extracted from the organ and then reinjected into the body, or in vivo, directly into the tissue in question. Being a negatively charged, high molecular weight molecule, DNA has difficulty in passing spontaneously through phospholipid cell membranes. Hence various vectors are used in order to permit gene transfer: viral vectors on the one hand, natural or synthetic chemical and/or biochemical vectors on the other hand. Chemical and/or biochemical vectors are, for example, cations (calcium phosphate, DEAE-dextran, etc.) which act by forming precipitates with DNA which can be "phagocytosed" by the cells. They can also be liposomes in which the DNA is incorporated and which

fuse with the plasma membrane. Synthetic gene transfer vectors are generally cationic polymers or lipids which complex DNA and form with the latter a particle carrying positive surface charges. These particles are capable of interacting with the negative charges of the cell membrane, and then of crossing the latter. Dioctadecylamidoglycylspermine (DOGS, Transfectam™) or N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA, Lipofectin™) may be mentioned as examples of such vectors. Chimeric proteins have also been developed; they consist of a polycationic portion which condenses DNA, linked to a ligand which binds to a membrane receptor and draws the complex into the cells by endocytosis. It is thus theoretically possible to "target" a tissue or certain cell populations so as to improve the in vivo bioavailability of the transferred gene (for reviews, see Behr, 1993, Cotten and Wagner, 1993). Among the viruses which are potentially usable as vectors for gene transfer, retroviruses (RSV, HMS, MMS, and the like), the HSV virus, adeno-associated viruses and adenoviruses may be mentioned more especially. These viruses have all been used to infect different cell types.

Gene therapy approaches have been developed for the treatment of various types of pathology, including nervous system disorders, cardiovascular diseases or cancer. As regards the cancer field more especially, various approaches have been proposed in

the prior art. Thus, some studies describe the use of lymphocytes activated ex vivo by culturing in the presence of interleukin-2 or by transfection with the interleukin-2 gene. Studies employing adoptive immunotherapy have also been undertaken with monocytes-macrophages purified and activated ex vivo with interferon in order to increase their tumoricidal power and then reinjected into patients (Andressen et al., Cancer Res. 50 (1990) 7450). The possibility of using genetically modified macrophages has also been described (WO95/06120). Another series of approaches is based on the transfer of toxic genes capable of inducing the death of cancer cells directly or indirectly. This type of approach has been described, for example, with the thymidine kinase gene, transferred in vivo either by an adenoviral vector (PCT/FR94/01284; PCT/FR94/01285) or by grafting cells that produce a retroviral vector (Caruso et al., PNAS 90 (1993) 7024). Other genes used are, for example, the cytosine deaminase gene.

The present application relates to a new method for the treatment of cancer. It is intended most especially for the treatment of human tumours, and in particular melanomas. The method of the invention is based on the in vivo transfer and expression of antigens specific to human tumours such as melanomas, capable of inducing (i) an immune protection against the appearance of this type of cancer, and (ii) an

expansion of the population of cytotoxic T cells (CTL) specific for cells possessing these antigens, and thus a destruction of the corresponding tumour cells by the immune system.

The immune system has, among other functions, the capacity to effect protection against viral infections. This capacity is discharged by cytotoxic T lymphocytes (CTL). CTL display two exceptional features: they are highly specific and of great efficacy. They destroy the infected cells after identifying a viral antigen at their surface. The antigen in question manifests itself in the form of a peptide combined with a major histocompatibility complex class I (MHC-I) molecule. In the context of tumours, if was observed, initially in mice, that these malignant cells possess peptide-MHC-I molecule complexes capable of producing, as in the context of antiviral responses, a CTL-mediated immune response. These peptides originate, in particular, from proteins encoded by genes which are mutated or activated selectively in the tumour cells. These proteins are designated tumour specific antigens. More recently, differentiation antigens recognized by CTL have been characterized on human tumours.

The present invention relates to a new method for the treatment of human tumours. It is the outcome, in particular, of the demonstration of vectors of viral origin capable of transferring and expressing in vivo

antigens specific to human tumours or to melanomas. It is based more especially on the demonstration in mouse models that defective recombinant adenoviruses are capable of inducing an immunization against this type of antigen, enabling lymphocytic responses to these antigens, and in particular tumour cells carrying them, to be obtained in vivo. This method according to the invention hence makes it possible, by the transfer of these genes, to act on the development of human tumours in an especially effective manner, stopping their progression, it being possible to bring about eradication.

A first subject of the invention hence lies in a defective recombinant adenovirus containing, inserted into its genome, a nucleic acid coding for a tumour-specific protein or peptide, and more especially for all or part of an antigen specific to a melanoma.

Preferably, the antigen in question is specific to a human melanoma. Still more preferably, it is a fragment of an antigen specific to a human melanoma comprising the portion presented to the CTL in combination with MHC-I molecules. The antigens specific to human tumours have been described by Thierry Boon et al., (US 5,342,774; US 5,405,940; WO92/20356; WO94/23031; WO94/21126). These antigens, designated by the term MAGE, are expressed selectively in tumour cells, mainly human tumours. Various human MAGE genes have been described, and in particular the genes

MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, MAGE-7, MAGE-8, MAGE-9, MAGE-10, MAGE-11 and MAGE-12. As a representative example of homologous mouse genes, the S-MAGE-1 and S-MAGE-2 genes may also be quoted. As regards, more especially, BAGE, GAGE and RAGE genes, these are representative of other families of related genes.

According to a preferred embodiment, the present invention relates to a defective recombinant adenovirus containing, inserted into its genome, a nucleic acid coding for a protein, or peptide derived from the latter, selected from the proteins Mage-1, Mage-3, Bage, Gage and Rage. These antigens are, in effect, the most selective, in the sense that they are not detected, for the most part, on any non-tumoral somatic cell. The sequence of the antigen Mage-1 and of the corresponding gene have been described, in particular, in Van der Bruggen et al., Science 254 (1991) 1644. The sequence of the cDNA coding for Mage-1 and Mage-3 has been described, for example, in Gaugler et al., (J. Exp. Med. 179 (1994) 921).

As stated above, a preferred embodiment of the invention is represented by a defective recombinant adenovirus containing, inserted into its genome, a nucleic acid coding for a peptide of the protein Mage-1. Mage-3, Bage or Gage comprising the portion presented to the CTL in combination with MHC-I molecules. The Mage, Bage and Gage genes code, in

effect, for large-sized proteins. These proteins are degraded by enzymatic digestion in the cell, leading to the generation of peptides. These peptides are the molecules which are then presented at the surface of the cells and which are recognized by the CTL in combination with MHC-I molecules (see Figure 2). Still more preferably, the invention relates to a recombinant adenovirus comprising, inserted into its genome, a nucleic acid coding for a peptide of the protein Mage-1 or Mage-3 comprising the portion presented to the CTL.

According to a specific embodiment, the invention relates to a recombinant adenovirus comprising, inserted into its genome, the sequence SEQ ID No. 1. This sequence comprises the sequence coding for the nonapeptide (27bp) of Mage-1 which is presented by the molecule HLA.A1 to the cytotoxic T lymphocytes. Still more preferably, the sequence in question is the sequence lying between residues 55 and 82 of the sequence SEQ ID No. 1.

According to another specific embodiment, the invention relates to a recombinant adenovirus comprising, inserted into its genome, the sequence SEQ ID No. 2. This sequence comprises the sequence coding for the nonapeptide (27bp) of Mage-3 which is presented by the molecule HLA.A1 to the cytotoxic T lymphocytes.

According to another embodiment, the invention relates to [lacuna] recombinant adenovirus

comprising, inserted into its genome, a nucleic acid coding for the antigenic peptide of the P1A gene of the DBA/2 mouse mastocytoma p815 (SEQ ID No. 3).

As stated above, the adenoviruses of the invention permit transfer and effective expression of these antigenic peptides in vivo. Thus, they make it possible, in a quite exceptional manner, to stimulate in vivo the appearance of cytotoxic T lymphocytes specific for these antigens, which selectively destroy any cell presenting this antigen at its surface.

Hence the viruses of the invention are usable for the preparation of pharmaceutical compositions intended for the treatment of cancers whose cells present Mage antigens at their surface. To prepare such compositions, a patient's tumour cells (generally from a melanoma) are preferably removed and analyzed in order (i) to determine the expression of a Mage gene for example, by RT-PCR, and (ii) where appropriate, to type this Mage antigen. An adenovirus containing a nucleic acid coding for all or part of the corresponding antigen is constructed and used for administration.

The viruses of the invention may also be used in vitro (or ex vivo) to generate populations of cytotoxic T cells specific for a given tumour antigen. To this end, a cell population is infected with a virus of the invention and then brought into contact with CTL cell precursors. The CTL cells specific for the

antigens may then be selected in vitro, amplified and thereafter used as a medicinal product in order to destroy the corresponding tumours specifically. Advantageously, the cell population infected with a virus of the invention comprises antigen presenting cells (APC). These may be in particular macrophages (WO95/06120) or B cells.

In the adenoviruses of the invention, the inserted nucleic acid may be a fragment of complementary DNA (cDNA) or of genomic DNA (gDNA), or a hybrid construction consisting, for example, of a cDNA into which one or more introns might be inserted. It can also comprise synthetic or semi-synthetic sequences. As stated above, the nucleic acid in question codes for a whole protein, or peptide derived from this protein, selected from Mage-1, Mage-3, Bage and Gage. For the purposes of the present invention, the expression peptide derived from this protein means that the nucleic acid can code for just a fragment of the protein, it being necessary for this fragment to be capable of generating CTL. The fragment according to the invention hence carries at least one antigenic determinant recognized by a specific CTL. These fragments may be obtained by any technique known to a person skilled in the art, and in particular by genetic and/or chemical and/or enzymatic modifications, or alternatively by cloning by expression, permitting the selection of variants in accordance with their

biological activity. Genetic modifications include suppressions, deletions, mutations, and the like.

The inserted nucleic acid is preferably a cDNA or from a gDNA.

Generally, the inserted nucleic acid also comprises sequences permitting the expression of the antigen or antigen fragment in the infected cell. The sequences can be ones which are naturally responsible for the expression of the said antigen when these sequences are capable of functioning in the infected cell. They can also be sequences of different origin, designated heterologous sequences (responsible for the expression of other proteins, or even synthetic sequences). In particular, the sequences can be promoters of eukaryotic or viral genes or derived sequences, stimulating or repressing the transcription of a gene specifically or non-specifically and inducibly or non-inducibly. As an example, they can be promoter sequences originating from the genome of the cell which it is desired to infect, or from the genome of a virus, and in particular the promoters of the adenovirus E1A and MLP genes, the CMV, RSV LTR, SRα promoter, and the like. Among eukaryotic promoters, there may also be mentioned the ubiquitous promoters (HPRT, vimentin, α-actin, tubulin, and the like), the promoters of intermediate filaments (desmin, neurofilaments, keratin, GFAP, and the like), the promoters of therapeutic genes (MDR, CFTR, factor VIII

type, and the like), tissue-specific promoters (pyruvate kinase, villin, intestinal fatty acid binding protein promoter, smooth muscle cell α-actin promoter, promoters specific for the liver; Apo AI, Apo AII, human albumin, and the like) or alternatively promoters responding to a stimulus (steroid hormone receptor, retinoic acid receptor, and the like). In addition, these expression sequences may be modified by the addition of activation, regulatory, and the like, sequences. Moreover, when the inserted nucleic acid does not contain expression sequences, it may be inserted into the genome of the defective virus downstream of such a sequence.

The viruses according to the present invention are defective, that is to say incapable of replicating autonomously in the target cell. Generally, the genome of the defective viruses used in the context of the present invention hence lacks at least the sequences needed for replication of the said virus in the infected cell. These regions may be either removed (wholly or partially), or rendered non-functional, or replaced by other sequences, and in particular by the inserted gene. Preferably, the defective virus nevertheless retains the sequences of its genome which are needed for encapsidation of the viral particles.

The viruses according to the invention may be obtained from different serotypes of adenovirus. Different serotypes of adenovirus exist, the structure

and properties of which vary somewhat. Among these serotypes, it is preferable to use, in the context of the present invention, human adenoviruses type 2 or 5 (Ad2 or Ad5) or adenoviruses of animal origin (see Application WO94/26914). Among adenoviruses of animal origin which are usable in the context of the present invention, adenoviruses of canine, bovine, murine (for example Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or alternatively simian (for example SAV) origin may be mentioned. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus [Manhattan or A26/61 (ATCC VR-800) strain, for example]. It is preferable to use adenoviruses of human or canine or mixed origin in the context of the invention.

Preferably, the defective adenoviruses of the invention comprise the ITRs, a sequence permitting encapsidation and the nucleic acid of interest. Still more preferably, in the genome of the adenoviruses of the invention, the E1 region at least is non-functional. The viral gene in question may be rendered non-functional by any technique known to a person skilled in the art, and in particular by total elimination, substitution, partial deletion or addition of one or more bases in the gene or genes in question. Such modifications may be obtained in vitro (on the isolated DNA) or in situ, for example by means of genetic engineering techniques or alternatively by

treatment by means of mutagenic agents. Other regions may also be modified, and in particular the E3 (WO95/02697), E2 (WO94/28938), E4 (WO94/28152, WO94/12649, WO95/02697) and L5 (WO95/02697) regions. According to a preferred embodiment, the adenovirus according to the invention comprises a deletion in the E1 and E4 regions. According to another preferred embodiment, it comprises a deletion in the E1 region, into which the E4 region and the nucleic acid are inserted (see FR94/13355). Advantageously, the deletion in the E1 region covers nucleotides 454 to 3328 (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Advantageously, the deletion in the E4 region comprises at least the frames ORF3 and ORF6.

The nucleic acid of interest may be inserted at different regions of the adenovirus genome. The genome of an adenovirus is composed of a linear double-stranded DNA approximately 36 kb in size. It comprises, in particular, an inverted repeat sequence (ITR) at each end, an encapsidation sequence (Psi), early genes and late genes (see Figure 1). The main early genes are contained in the E1, E2, E3 and E4 regions. Among these genes, those contained in the E1 region are needed for viral propagation. The main late genes are contained in the L1 to L5 regions. The genome of the Ad5 adenovirus has been completely sequenced and is accessible on a database (see, in particular, Genebank M73260). Similarly, portions or even the whole of other

adenoviral genomes (Ad2, Ad7, Ad12, and the like) have also been sequenced. The nucleic acid of interest is preferably inserted into a region which is not essential to the production of the defective recombinant viruses. Thus, it is preferably inserted into the E1 region, which is defective in the virus and complemented by the producing line, into the E3 region, which is not essential to the production of the recombinant viruses (its inactivation does not need to be transcomplemented), or alternatively into the E4 region. In the latter case, it is necessary to complement the E4 functions during production, either by cotransfection with a helper virus or plasmid, or by means of a suitable line. Clearly, other sites may be used. In particular, access to the nucleotide sequence of the genome enables a person skilled in the art to identify regions enabling the nucleic acid of interest to be inserted.

The defective recombinant adenoviruses according to the invention may be prepared by any technique known to a person skilled in the art (Levrero et al., Gene 101 (1991) 195, EP 185 573; Graham, EMBO J. 3 (1984) 2917). Generally, the adenoviruses are produced by transfection of the DNA of the recombinant virus into a competent encapsidation cell line. The transfection may be a single one, when it is possible to have at one's disposal a construction carrying the whole of the genome of the recombinant virus, or, as is

most often the case, a cotransfection of several DNA fragments supplying the different portions of the recombinant viral genome. In this case, the process involves one or more steps of homologous recombination between the different constructions in the encapsidation cell line, in order to generate the DNA of the recombinant virus. The different fragments used for the production of the virus may be prepared in different ways. The technique most generally used consists in isolating the viral DNA and then in modifying it in vitro by the standard methods of molecular biology (digestion, ligation, and the like). The constructions obtained are then purified and used to transfect the encapsidation lines. Another technique is based on the use of a plasmid carrying a portion of the genome of the recombinant virus, which is cotransfected with a virus supplying the missing portion of the genome. Another possibility lies in the use of prokaryotic plasmids to prepare the viral DNAs which are usable for the transfection (see Bett et al., PNAS 91 (1994) 8802, FR95/01632).

The cell line used should preferably (i) be transformable by the said elements, and (ii) contain the sequences capable of complementing the portion of the genome of the defective adenovirus, preferably in integrated form in order to avoid risks of recombination. As an example of a line, there may be mentioned the human embryonic kidney line 293 (Graham

et al., J. Gen. Virol. 36 (1977) 59) which contains, in particular, integrated in its genome, the left-hand portion of the genome of an Ad5 adenovirus (12 %) or lines capable of complementing the E1 and E4 functions, as are described, in particular, in Applications Nos. WO94/26914 and WO95/02697.

Thereafter, the adenoviruses which have multiplied are recovered and purified according to the standard techniques of molecular biology, as illustrated in the examples.

The present invention also relates to any pharmaceutical composition comprising one or more defective recombinant adenoviruses as described above. The pharmaceutical compositions of the invention may be formulated for the purpose of oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, transdermal, intratracheal, intraperitoneal, and the like, administration.

The present invention also relates to any pharmaceutical composition comprising cells infected with a defective recombinant adenovirus as described above. Advantageously, the composition of the invention comprises antigen presenting cells (APC) infected with a defective recombinant adenovirus as described above. As a specific example, there may be mentioned macrophages or B lymphocytes. The invention also relates to a composition comprising tumour antigen-specific cytotoxic T cells (CTL) prepared by culturing

precursor cells in the presence of antigen presenting cells (APC) infected with a defective recombinant adenovirus as described above.

Preferably, a pharmaceutical composition of the invention contains vehicles which are pharmaceutically acceptable for an injectable formulation. These can be, in particular, sterile, isotonic saline solutions (containing monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, in particular lyophilized, compositions which, on adding sterilized water or physiological saline, as the case may be, enable injectable solutions to be made up.

The doses of virus used for injection may be adapted in accordance with different parameters, and in particular in accordance with the mode of administration used, the pathology in question, the gene to be expressed or alternatively the desired period of treatment. Generally speaking, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu, and preferably of 10^6 to 10^{10} pfu. The term pfu (plaque forming unit) corresponds to the infectious power of a solution of virus, and is determined by infecting a suitable cell culture and measuring, generally after 15 days, the number of plaques of infected cells. The techniques of

determination of the pfu titre of a viral solution are well documented in the literature.

Depending on the antigen in question, the adenoviruses of the invention may be used for the treatment or prevention of cancer, including, in particular, human tumours (for the antigens Mage-1 to Mage-12, Gage and Bage and Rage) and sarcomas (for the Mage-1 antigens).

The present invention will be described more completely by means of the examples which follow, which are to be regarded as illustrative and non-limiting.

LEGEND TO THE FIGURES

Figure 1: Genetic organization of the Ad5 adenovirus.

Figure 2: Expression and processing of the Mage antigens.

Figure 3: Construction of the plasmids pAd.SRα-MAGE.

Figure 4: Protocol No. 1 for immunization of DBA/2 mice with an Ad-P1A or control.

Figure 5: Protocol No. 2 for immunization of DBA/2 mice with an Ad-P1A or control.

Table 1: Demonstration of the specific lysis by CTL of cells infected with an Ad-Mage according to the invention.

Table 2: Demonstration of the capacity of cells infected with an Ad-Mage according to the invention to stimulate the production of TNF by a CTL clone.

Table 3: Demonstration of the immunization of DBA/2

mice by injection, according to Protocol 1, of an Ad-P1A (Table 3A) or of a control adenovirus, Ad-βGal (Table 3B).

Table 4: Demonstration of the immunization of DBA/2 mice by injection, according to Protocol 2, of an Ad-P1A (Table 4A) or of a control adenovirus, Ad-βGal (Table 4B)

General techniques of cloning and of molecular biology

The traditional methods of molecular biology, such as centrifugation of plasmid DNA in a caesium chloride/ethidium bromide gradient, digestion with restriction enzymes, gel electrophoresis, transformation in E.coli, precipitation of nucleic acids and the like, are described in the literature (Maniatis et al., 1989).

Enzymes were supplied by New England Biolabs (Beverly, MA).

To carry out ligation, the DNA fragments are separated according to their size on 0.8 to 1.5 % agarose gels, purified with GeneClean (BI0101, La Jolla CA) and incubated overnight at 14°C in a buffer comprising 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, in the presence of phage T4 DNA ligase.

Amplification by PCR (polymerase chain reaction) was also carried out according to Maniatis et al., 1989, with the following specifications:

- $MgCl_2$ concentration brought to 8 mM;

- Denaturation temperature 95°C, hybridization temperature 55°C, elongation temperature 72°C. This cycle was repeated 25 times in a PE9600 Thermalcycler (Perkin Elmer, Norwalk CO).

Oligonucleotides are synthesized using phosphoramidite chemistry in which the phosphoramidites are protected at the β position with a cyanoethyl group (Sinha et al., 1984, Giles 1985), with an Applied Biosystem model 394 automatic DNA synthesizer (Applied Biosystem, Foster City CA), according to the manufacturer's recommendations.

Sequencing was performed on double-stranded templates by the chain termination method using fluorescent primers. We used the Taq Dye Primer Kit sequencing kit from Applied Biosystem (Applied Biosystem, Foster City CA) according to the manufacturer's specifications.

Example 1: Construction of a defective recombinant adenovirus coding for a P1A antigen fragment

This example describes the construction of a defective recombinant adenovirus according to the invention coding for a fragment of the antigen P1A. More especially, the adenovirus carries the sequence SEQ ID No. 3. The adenovirus constructed is an adenovirus of serotype 5, possessing a deletion in the E1 and E3 regions, the nucleic acid of interest being inserted into the E1 region, at the level of the

deletion.

The nucleic acid inserted into the E1 region comprises more especially:

- the SRα promoter. The SRα promoter comprises the early origin of replication of SV40 and a portion of the HTLV1 LTR (corresponding to the domain R and to a portion of U5), followed by the 16S splice junction of SV40 (Takebe et al., Mol. Cell. Biol. 8 (1988) 466).
- a P1A minigene of 44 bp (SEQ ID No. 3).
- the polyadenylation site of the SV40 virus.

This nucleic acid was extracted from the plasmid pcD-SRα-P1A, corresponding to the plasmid pcD-SRα into which the P1A minigene has been cloned at the EcoRI site. The insert obtained was then cloned into the plasmid pAd.RSV-βGal (Stratford-Perricaudet et al., J. Clin. Invest. 90 (1992) 626), in place of the fragment containing the RSV LTR and the LacZ gene.

The plasmid pAd-SRα-P1A thereby obtained was then used to produce the recombinant adenovirus. To do this, line 293 cells were cotransfected with 5 µg of plasmid pAd-SRα-P1A and with 5 µg of the DNA of the mutant adenovirus dl 324 in the presence of calcium phosphate. The recombinant adenoviruses produced were then selected by plaque purification. After isolation, the recombinant adenovirus is amplified in the cell line 293, leading to a culture supernatant containing

the unpurified recombinant adenovirus having a titre of approximately 10^{10} pfu/ml.

The viral particles are then purified by centrifugation on a caesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). Analysis of the viral DNA by digestion using EcoRI restriction enzymes demonstrates the presence of the insert in the genome. The adenovirus may be stored at -80°C in 10% glycerol.

Example 2: Construction of a defective recombinant adenovirus coding for a Mage-1 antigen fragment

This example describes the construction of a defective recombinant adenovirus according to the invention coding for a fragment of the antigen Mage-1. More especially, the adenovirus carries the sequence SEQ ID No. 1 coding for a fragment carrying the antigenic nonapeptide of Mage-1. The adenovirus constructed is an adenovirus serotype 5, possessing a deletion in the E1 and E3 regions, the nucleic acid of interest being inserted into the E1 region where the deletion is present.

The nucleic acid inserted into the E1 region comprises, more especially:

- the SRα promoter. The SRα promoter comprises the early origin of replication of SV40 and a portion of the HTLV1 LTR (corresponding to the domain R and to a portion of U5), followed by the 16S splice

junction of SV40 (Takebe et al., Mol Cell Biol 8 (1988) 466).

- a MAGE-1 minigene of 116 bp (SEQ ID No. 1). This fragment was obtained by PCR from the complete Mage-1 gene. It contains an ATG at position 15, a stop codon at position 121 and a portion of exon 3 of the Mage-1 gene. It comprises the sequence corresponding to the nonapeptide (27 bp) which is presented by the HLA.A1 molecule to the cytotoxic T lymphocytes (see Figure 2).

- the polyadenylation site of the SV40 virus.

This nucleic acid was extracted from the plasmid pcD-SRα-MAGE-1, corresponding to the plasmid pcD-SRα into which the Mage-1 minigene has been cloned at the EcoRI site. The insert obtained was then cloned into the plasmid pAd.RSV-βGal (Stratford-Perricaudet et al., J. Clin. Invest. 90 (1992) 626), in place of the fragment containing the RSV LTR and the LacZ gene (Figure 3).

The plasmid pAd-SRα-MAGE-1 thereby obtained was then used to produce the recombinant adenovirus. To this end, line 293 cells were cotransfected with plasmid pAd-SRα-MAGE-1 and with the DNA of the mutant adenovirus dl 324 in the presence of calcium phosphate. The recombinant adenoviruses produced were then selected by plaque purification. After isolation, the recombinant adenovirus is amplified in the cell line 293, leading to a culture supernatant containing the

unpurified recombinant adenovirus having a titre of approximately 10^{10} pfu/ml.

The viral particles are then purified by centrifugation on a caesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). Analysis of the viral DNA by digestion using EcoRI restriction enzymes demonstrates the presence of the insert in the genome. The adenovirus may be stored at -80°C in 10 % glycerol.

Example 3: Construction of a defective recombinant adenovirus coding for a Mage-3 antigen fragment

This example describes the construction of a defective recombinant adenovirus according to the invention coding for a fragment of the antigen Mage-3. More especially, the adenovirus carries the sequence SEQ ID No. 2 coding for a fragment carrying the antigenic nonapeptide of Mage-3. The adenovirus constructed is an adenovirus serotype 5, possessing a deletion in the E1 and E3 regions, the nucleic acid of interest being inserted into the E1 region where the deletion is present.

The nucleic acid inserted into the E1 region comprises, more especially:

- the SRα promoter. The SRα promoter comprises the early origin of replication of SV40 and a portion of the HTLV1 LTR (corresponding to the domain R and to a portion of U5), followed by the 16S splice

junction of SV40 (Takebe et al., Mol. Cell. Biol. 8 (1988) 466).

- a MAGE-3 minigene of 44 bp (SEQ ID No. 2). This fragment was obtained by PCR from the complete Mage-3 gene. It contains an ATG at position 12, a stop codon at position 44 and the sequence corresponding to the nonapeptide (27 bp) which is presented by the HLA.A1 molecule to the cytotoxic T lymphocytes (see Figure 2).

- the polyadenylation site of the SV40 virus.

This nucleic acid was extracted from the plasmid pcD-SRα-MAGE-3, corresponding to the plasmid pcD-SRα into which the Mage-3 minigene has been cloned at the EcoRI site. The insert obtained was then cloned into the plasmid pAd.RSV-βGal in place of the fragment containing the RSV LTR and the LacZ gene (Figure 3).

The plasmid pAd-SRα-MAGE-3 thereby obtained was then used to produce the recombinant adenovirus. To this end, line 293 cells were cotransfected with plasmid pAd-SRα-MAGE-3 and with the DNA of the mutant adenovirus d1324 in the presence of calcium phosphate. The recombinant adenoviruses produced were then selected by plaque purification. After isolation, the recombinant adenovirus is amplified in the cell line 293, leading to a culture supernatant containing the unpurified recombinant adenovirus having a titre of approximately 10^{10} pfu/ml.

The viral particles are then purified by

centrifugation on a caesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). Analysis of the viral DNA by digestion using EcoRI restriction enzymes demonstrates the presence of the insert in the genome. The adenovirus may be stored at -80°C in 20 % glycerol.

Example 4: Functional characterization of the adenoviruses of the invention

This example demonstrates that the viruses according to the invention are capable of inducing the expression of the gene of interest coding for a protein whose degradation leads to the expression of an antigenic peptide at the surface of the target cells.

The expression of the MAGE-1 minigene and the presentation of the peptide were demonstrated on cells infected with Ad-Mage (4.1.), by determination of specific lysis (4.2.) and stimulation of TNF production (4.3.).

4.1. Cell lines

The cell lines which have been infected are the following:

- C1R.A1: B lymphocyte line transformed with EBV (ref. Storkus, W.J., Howell, D.N., Salter, R.D., Dawson, J.R., and Cresswell, P.: NK susceptibility varies inversely with target cell class I HLA antigen expression. J. Immunol. 138: 1675-1659, 1987) and transfected with the HLA.A1 gene cloned into the

plasmid pHEBO.

- Gerl III β E^-F^-: HLA.A1 human melanoma cells immunoselected for the loss of the antigen MAGE-1 (designated Gerlach E^- in Tables 1 and 2).

Hence these two lines express the HLA.A1 molecule but not the antigen MAGE-1.

4.2. Determination of specific lysis

This example demonstrates the existence of a specific lysis of the cells by a CTL clone specific for the antigen (radioactive chromium release test). To this end, the cells mentioned in 4.1. were infected with the adenovirus Ad-Mage-1 (Example 2) or with a control adenovirus (Ad-βGal) at a multiplicity of infection of 500 pfu/cell. The infected cells were then labelled with chromium-51 and thereafter incubated for 4 hours, on the basis of 1000 cells/well, with the specific CTL (clone 82:30) at different effector cells/target cell (E/T) ratios. The percentage lysis was then determined. The results obtained are presented in Table 1. They show clearly that cells infected with the viruses according to the invention display a sensitivity to lysis by the specific CTL which is markedly greater than that of cells infected with the control adenovirus. These cells also display a markedly enhanced sensitivity relative to cells transfected directly with the antigen Mage-1, thereby demonstrating the therapeutic efficacy of the vectors of the invention.

Hence these results show clearly that the viruses of the invention are capable of endowing cells with a considerable sensitivity to lysis by the specific CTL.

4.3. Stimulation of TNF production

In this example, the capacity of Gerl III β E^-F^- cells to stimulate the production of TNF by the same CTL clone was evaluated. To this end, Gerl III β E^-F^- cells were infected with the adenovirus Ad-Mage-1 (Example 2) or with a control adenovirus (Ad-β-Gal) at a multiplicity of infection of 50 or 100 pfu/cell, and then incubated with the CTL clone. After 24 hours, the amount of TNF present in the supernatants was measured by determination of their cytotoxicity on a TNF-sensitive line (line WEHI-164-13). Cell viability was measured by means of a colorimetric test (MTT). The results are expressed as optical density and then as quantity of TNF (pg/ml). This test was not performed on C1R.A1 cells, since they secrete TNF naturally. The Gerlach E^+ control cells are melanoma cells expressing Mage-1 and HLA-A1.

The results obtained are presented in Table 2. They show clearly that the cells infected with the viruses according to the invention induce a production of TNF by the CTL, thereby confirming unambiguously the biological and therapeutic properties of the viruses of the invention.

Example 5: In vivo activity of the P1A viruses of the invention

Example 4 showed that, in vitro or ex vivo, cells infected with an adenovirus according to the invention are indeed recognized in a TNF test, and lysed in a test of radioactive chromium release by a specific CTL.

This example now demonstrates that, in vivo, the adenoviruses according to the invention are capable of generating a specific CTL response. More especially, this example demonstrates that 2 injections one week apart of 10^9 viral particles (pfu) into DBA/2 mice generate a strong specific CTL response in a portion of them.

Two series of experiments were carried out. The plans of these two series are presented in Figures 4 and 5. In the first series of experiments (see plan Figure 4), half of the viral particles were administered into the peritoneal cavity and the other half under the skin (at 4 sites). In the second series of experiments (see plan Figure 5), the viral particles were administered by subcutaneous, intraperitoneal, intranasal and intratracheal injections. These two series of experiments were carried out according to the following protocol. The mice were sacrificed 15 days after the second series of injections of the adenovirus. The spleen cells of these mice were then brought into contact with the antigen P815A by means of

L1210A+ cells (syngeneic leukaemia transfected with the P_1A gene of the P815 mastocytoma) irradiated for a period of 8 days. On completion of this mixed lymphocyte-tumour culture (MLTC), lymphocytes directed against the antigen P815A have proliferated and have differentiated into killer T lymphocytes. The latter are then brought into contact with cells labelled with radioactive chromium. The cells in question are P511 cells, an azaguanine-resistant variant of the P815 mouse mastocytoma carrying the A antigen, and P1.204 cells, a variant of this same mastocytoma which has lost the A antigen. The latter serves as a negative control. In order to eliminate all possibility of non-specific reaction, the target cells labelled with radioactive chromium are also brought into contact with syngeneic cells (L1210) carrying at their surface the antigens of the stimulating cell in the MLTC, with the exception of the A antigen of P815.

The results obtained with the first series of experiments, expressed as a percentage of specific lyses, are presented in Tables 3A and 3B.

The results obtained with the second series of experiments, expressed as a percentage of specific lyses, are presented in Tables 4A and 4B.

In both cases, the results presented show, mouse by mouse, levels of lysis which are proportional to effectors/targets ratios (means of duplicates). CTLs were obtained whatever the site of injection of the

adenovirus (Table 4A + B). These results show clearly that the adenoviruses of the invention are capable of generating in vivo an immunity against cells carrying the tumour antigen.

Table 1

E/T ratio	C1R infected Ad Mage-1	C1R infected Ad β gal	C1R trans-fected with Mage-1	Gerlach E-infected Ad Mage-1	Gerlach E-infected Ad β gal
30	62	3	33	15	0
10	59	2	40	15	0
3	61	4	45	13	0
1	44	3	30	6	0
0.3	44	1	25	5	0
0.1	28	2	19	2	2
spontaneous	143	173	209	860	1049
maximum	1563	1958	1618	8320	7334
spont/max	9 %	9 %	13 %	10 %	14 %
% of blue cells		40%			100 %
pfu/c	500	500		500	500

100 µl super-natant

Table 2

				P82:30 Anti-Mage-1 CTL clone -CTL	+CTL
Gerlach			pfu/cell		
	Gerlach E+	(HLA A1+) (Mage 1+)		1.049 = 0 pg TNF	0.17 = 45 pg TNF
	Gerlach E-	(HLA A1+) (Mage 1-) infected with Ad. βgal	50	1.082 = 0 pg TNF	0.995 = 0 pg TNF
	Gerlach E-	(HLA A1+) (Mage 1-) infected with Ad. βgal	100	1.045 = 0 pg TNF	0.98 = 0 pg TNF
	Gerlach E-	(HLA A1+) (Mage 1-) infected with Ad. Mage 1	50	1.015 = 0 pg TNF	0.58 = 4 pg TNF
	Gerlach E-	(HLA A1+) (Mage 1-) infected with Ad. Mage 1	100	1.028 = 0 pg TNF	0.355 = 12 pg TNF
Controls	Medium			1.102 = 0 pg TNF	
	CTL P62.30				1.086 = 0 pg TNF
% of blue cells		Gerlach : 40 to 50 % with 50 pfu and 60 to 70 % with 100 pfu			

Table 3A

1°: mice injected with the recombinant adenovirus P1A

	effector /target	P511	P1.204	L1210A+ + cold L1210	L1210 + cold L1210	L1210A+	L1210
				(50 cold targets per labelled target)			
Mouse No. 1	218	14	10	4	0	15	21
	71	6	0	3	2	7	13
	24	5	0	0	0	6	1
	8	2	0	0	0	3	11
	3	4	2	0	0	0	5
	1	2	0	0	0	6	0
Mouse No. 2	325	77	44	100	30	96	74
	108	83	36	78	0	96	65
	36	69	24	56	1	78	50
	12	59	14	29	0	47	35
	4	37	3	3	4	23	20
	1	14	0	0	0	8	19
Mouse No. 3	250	76	34	64	10	81	92
	83	63	34	41	8	88	65
	28	42	23	13	5	59	54
	9	25	8	1	0	13	24
	3	14	1	0	0	6	1
	1	2	0	0	0	1	8
Mouse No. 4	550	66	45	77	25	85	66
	183	55	40	42	20	74	41
	61	41	28	22	2	47	40
	20	25	7	7	1	27	43
	7	12	7	0	0	22	16
	2	6	2	1	0	5	6

Table 3B

2°: mice injected with the recombinant adenovirus βgal

	effector/ target	P511	P1.204	L1210A+ + cold L1210	L1210 + cold L1210	L1210A+	L1210
				(50 cold targets per labelled target)			
Mouse No. 1	375	1	2	2	0	11	20
	125	5	5	0	0	6	2
	42	3	0	1	0	1	6
	14	4	0	0	0	1	3
	5	2	1	0	0	0	1
	2	0	0	0	0	0	1
Mouse No. 2	463	11	0	10	0	12	14
	154	6	0	0	0	10	0
	51	5	3	0	0	2	1
	17	2	0	0	0	3	7
	6	2	0	1	2	2	0
	2	3	0	0	0	0	5
Mouse No. 3	438	4	0	0	0	8	12
	146	7	2	0	0	1	11
	49	0	0	5	0	0	7
	16	2	0	0	0	3	4
	5	0	0	0	0	0	1
	2	2	0	0	0	0	0
Mouse No. 4	488	6	2	2	0	13	4
	163	8	1	0	0	0	0
	54	6	0	0	0	2	11
	18	1	0	1	0	3	0
	6	4	0	0	0	2	4
	2	2	0	0	0	0	6
	spontaneous	236	194	140	116	125	95
	maximum	1722	1170	786	547	790	449
	spont/max	14 %	16 %	10 %	21 %	16 %	21 %

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Table 4a

		Effector/ target	P511	P1.204	P511 + cold L1210 50><1 ratio	P1.204 + cold L1210 50><1 ratio
Subcutaneous						
	Mouse	490	9	2	0	1
	No.1	163	5	3	0	0
		54	3	1	0	0
		18	3	0	0	0
		8	2	1	0	0
		2	0	0	0	0
					0	
	Mouse	50	5	0	0	1
	No.2	27	3	0	3	0
		9	5	0	0	0
		3	6	0	1	0
		1	4	0	0	0
		0.3	2	0		0
					68	
	Mouse	300	73	18	72	0
	No.3	100	71	15	75	0
		33	92	10	57	1
		11	73	8	25	8
		4	56	3	8	0
		1	25	2		0

Table 4a (continued)

		Effector/ target	P511	P1.204	P511 + cold L1210 50><1 ratio	P1.204 + cold L1210 50><1 ratio
Intra-peritoneal						
	Mouse No.1	258	51	9	48	0
		85	43	10	37	0
		28	43	8	27	0
		9	38	3	12	1
		3	18	2	8	0
		1	7	0	1	0
	Mouse No.2	295	9	4	9	2
		88	8	6	2	0
		33	7	4	2	0
		11	3	2	1	0
		4	1	2	0	0
		1	0	0	0	1
	Mouse No.3	400	68	12	76	0
		133	77	12	82	0
		44	88	14	73	0
		15	14	7	66	0
		6	10	6	30	0
		3	10	1	14	0
	Mouse No.4	185	8	2	3	0
		52	3	0	3	0
		57	10	2	3	0
		8	4	1	2	0
		2	3	0	0	0
		0.8	2	0	2	0

Table 4a (continued)

		Effector/ target	P511	P1.204	P511 + cold L1210 50><1 ratio	P1.204 + cold L1210 50><1 ratio
Intratracheal						
	Mouse No.1	440	67	11	50	3
		147	78	13	52	2
		49	64	6	49	0
		18	34	8	23	0
		6	31	7	9	0
		2	14	0	2	0
	Mouse No.2	410	8	7	3	2
		137	6	7	3	1
		45	6	4	0	1
		15	5	2	2	0
		9	1	3	0	0
		2	1	2	0	2
	Mouse No.3	344	8	2	3	1
		115	4	0	2	0
		38	8	0	1	0
		13	8	0	0	0
		4	4	1	0	0
		1	1	0	0	0

Table 4a (continued)

Mouse No.4	265	80	9	52	0	Mouse No.5	230	2	0	1	0
	85	46	8	47	0		77	4	0	3	0
	28	42	7	21	0		25	2	0	0	0
	9	33	3	9	0		8	2	0	0	0
	3	17	2	2	0		3	1	1	0	0
	1	5	2	1	0		1	1	2	0	0
Mouse No.6	210	13	2	11	1						
	70	10	0	7	0						
	23	6	0	2	0						
	3	1	0	0	0						
	3	2	0	0	0						
	1	2	0	0	0						

Table 4a (continued)

Intranasal	Mouse No.1	430	58	0	52	0	6	1	0	0	0	0	2	0	0	0	0	0
		160	52	0	58	0												
		53	84	4	65	0												
		18	84	2	52	0												
		6	51	2	33	0												
		2	52	0	20	0												
	Mouse No.2	205	4	0	0	0												
		69	4	0	0	0												
		83	1	0	0	0												
		8	5	0	0	0												
		2	3	0	0	0												
		0.8	2	0	0	0												

Table 4a (continued)

		Effector/ target	P511	P1.204	P511 + cold L1210 50><1 ratio	P1.204 + cold L1210 50><1 ratio
Intranasal						
	Mouse No.3	250	1	0	0	0
		83	0	0	0	0
		28	1	0	0	0
		9	1	0	0	0
		3	1	0	0	0
		1	1	0	0	0
	Mouse No.4					
		280	2	0	1	0
		87	8	1	0	0
		29	1	0	0	0
		10	2	0	1	0
		3	0	0	0	0
	Mouse No.5	1	1	1	0	0
		240	3	0	0	2
		80	1	0	0	0
		27	1	0	0	1
		9	1	1	0	0
	Mouse No. 6	3	1	0	0	0
		1	1	1	1	0
		125	0	0	0	0
		42	0	0	0	0
		14	0	0	0	0
		5	1	0	0	0
		2	0	0	0	1
		0.6	0	0	0	0

Table 4b

2°: Adβgal injected mice

		Effector/ target	P511	P1.204	P511 + cold L1210 50><1 ratio	P1.204 + cold L1210 50><1 ratio
Subcutaneous						
	Mouse No.1	300	0	0	0	0
		100	0	0	0	0
		33	0	0	0	0
		11	0	0	0	0
		4	0	0	0	1
		1	0	0	0	0
	Mouse No.2	230	4	3	0	1
		77	1	1	0	0
		25	1	4	0	0
		8	0	0	0	0
		3	0	0	0	0
		1	0	0	0	0
Intra-peritoneal						
	Mouse No.1	220	1	2	1	0
		73	0	0	0	1
		24	1	1	1	2
		8	0	0	0	0
		3	0	0	0	1
		1	0	0	0	0
	Mouse No.2	410	9	1	0	3
		137	1	1	0	2
		45	0	0	0	0
		15	0	1	0	0
		5	0	1	0	0
		2	0	0	0	0

Table 4b (continued)

Intra-tracheal						
	Mouse No.1	310	0	0	0	0
		103	0	0	0	0
		34	0	0	0	0
		11	0	0	0	0
		4	0	0	0	0
		1	0	0	0	0
	Mouse No.2	270	2	1	0	1
		90	1	0	0	0
		30	0	0	0	0
		10	0	0	0	0
		3	1	0	0	0
		1	1	0	0	0
Intranasal						
	Mouse No.1	400	0	0	0	0
		18	1	0	0	1
		5	0	0	0	0
		2	0	0	0	0
	Mouse No.2	230	3	0	0	1
		77	2	0	0	0
		25	0	0	0	0
		9	0	0	0	0
		3	1	0	0	0
		1	0	0	0	0
		spontaneous	106	127	122	110
		maximum	1041	971	957	988
		spont/max	10 %	13 %	13 %	12 %

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(F) POSTAL CODE: 10105

(ii) TITLE OF INVENTION: METHOD FOR THE TREATMENT OF HUMAN TUMOURS BY GENE THERAPY AND CORRESPONDING RECOMBINANT VIRUSES.

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Tape

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 126 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AAGGAAGCAG ACCCCACCGG CCACTCCTAT GTCCTTGTCA CCTGCCTAGG 100

TCTCTCCTAT GATGGCTAGA ATTCTT 126

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCGCCGC CATGGAAGTG GACCCCATCG GCCACTTGTA CTAG 44

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AATTCGCCGC CATGCTGCCT TATCTAGGGT GGCTGGTCTT CTAG 44

CLAIMS

1. Defective recombinant adenovirus containing, inserted into its genome, a nucleic acid coding for a tumour-specific protein or peptide capable of inducing an immune protection and a destruction of the corresponding tumour cells by the immune system.

2. Defective recombinant adenovirus according to claim 1, characterized in that it contains a nucleic acid coding for a protein or peptide specific to a human tumour.

3. Defective recombinant adenovirus according to claim 1 or 2, characterized in that the nucleic acid inserted into its genome codes for all or part of an antigen specific to a melanoma.

4. Adenovirus according to claim 3, characterized in that the nucleic acid in question codes for a fragment of an antigen specific to a human melanoma comprising the portion presented to the CTL in combination with MHC-I molecules.

5. Adenovirus according to one of the preceding claims, characterized in that the nucleic acid codes for a protein, or a peptide derived therefrom, selected from the proteins Mage-1, Mage-3, Bage, Rage and Gage.

6. Defective recombinant adenovirus comprising, inserted into its genome, a nucleic acid coding for a peptide of the protein Mage-1 or Mage-3 comprising the portion presented to the CTL.

7. Defective recombinant adenovirus comprising, inserted into its genome, the sequence SEQ ID No. 1.

8. Defective recombinant adenovirus comprising, inserted into its genome, the sequence lying between residues 55 and 82 of the sequence SEQ ID No. 1.

9. Defective recombinant adenovirus comprising, inserted into its genome, the sequence SEQ ID No. 2.

10. Adenovirus according to one of the preceding claims, characterized in that it is chosen from the human serotypes Ad2 and Ad5.

11. Adenovirus according to one of claims 1 to 9, characterized in that it is chosen from canine serotypes.

12. Adenovirus according to one of the preceding claims, characterized in that it contains a deletion in the E1 region.

13. Adenovirus according to claim 11, characterized in that it contains, in addition, a deletion in the E4 region.

14. Adenovirus according to one of the preceding claims, characterized in that the nucleic acid is inserted into the E1 or E3 or E4 region.

15. Pharmaceutical composition comprising at least one adenovirus according to one of the preceding claims.

16. Use of an adenovirus according to one of claims 1 to 14, for the in vitro or ex vivo production of cytotoxic lymphocytes specific for human tumours.

17. Composition comprising cells infected with a defective recombinant adenovirus according to one of claims 1 to 14.

18. Composition according to claim 17, characterized in that it comprises antigen presenting cells (APC) infected with a defective recombinant adenovirus according to one of claims 1 to 14.

19. Method of preparing cytotoxic T cells specific for a tumour antigen comprising bringing a CTL cell precursor into contact with a population of cells infected with a virus according to one of claims 1 to 14.

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Abstract

A method for treating human tumours by gene therapy is disclosed. In particular, defective recombinant viruses with a sequence coding for a human tumour-specific antigen, and the use thereof for treating or preventing human tumours, as well as producing specific cytotoxic T-cells (CTLs) in vitro or ex vivo, are disclosed. Pharmaceutical compositions comprising said viruses, particularly in injectable form, are also disclosed.

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

RECOMBINANT ADENOVIRAL VECTORS FOR HUMAN TUMOUR GENE THERAPY

the international specification of which was filed on March 12, 1997 as Application Serial No. PCT/FR97/00435 which notice of transmission was given on September 18, 1997, by the International Bureau. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of a foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications(s)			Priority Claimed	
FR96/03207	France	14 March 1996	X	
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)
(Application Serial No.)	(Filing Date)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint the attorneys associated with the Customer Number provided below as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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